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# Phenotypic Variability of Filipino βº-Thalassemia/HbE Patients in Indonesia

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**Three Indonesian patients with identical genotypes, each compound heterozygotes for Filipino βº-thalassemia/HbE, expressed different clinical severities. One patient has mild disease and is transfusion independent, while the other two are severely affected and transfusion dependent. The size of the Filipino βº-globin gene deletion was confirmed to be 45 kb, resolving conflicting values given in the literature. Neither ameliorating genetic factors such as α-globin gene deletions or the *Xmn*I restriction site polymorphism at position -158 upstream of the $^{G}\gamma$-globin gene, nor differences in β-globin gene haplotype, explain the phenotypic variation. These observations have implications for the development of antenatal diagnosis in Indonesia, as at present it is not possible to give an accurate prediction of severity of phenotype for this common genotype. Am. J. Hematol. 62:7–12, 1999.** 

**Key words: β-thalassemia/HbE; phenotype-genotype; deletion**

## INTRODUCTION

The phenotypic diversity found in β-thalassemia/HbE patients has recently been reviewed [1,2]. Possible explanations for the observed variable clinical severity are: (1) heterogeneity of β-thalassemia mutations [3]; (2) coinheritance of α-thalassemia [4]; or (3) level of expression correlated with specific β-globin gene haplotypes and the presence or absence of the *Xmn*I restriction site polymorphism at position -158 in the $^{G}\gamma$-globin gene associated with high levels of synthesis of fetal hemoglobin [5–8]. A recent study suggested a fourth explanation, that the extent of mis-splicing of the $\beta^{E}$-globin mRNA in β-thalassemia/HbE patients gives a better correlation with clinical severity than the coinheritance of α-thalassemia or the *Xmn*I-$^{G}\gamma$ polymorphism [9].

The Filipino βº-thalassemia deletion was first reported by Motum et al. [10], and later by Eng et al. [11] and Dimovski et al. [12]. Carriers have an unusually high $HbA_2$ level. All studies reported the same 5′ breakpoint of the deletion, but the 3′ breakpoint apparently differed as the size of the deletion ranged from 45 kb [10] to 105 kb [12].

We report three cases of Filipino βº-thalassemia/HbE showing different phenotypes, from mild to severe, and ameliorating factors were investigated to explain this phenotypic diversity. The size of the Filipino deletion in the β-globin gene was defined by pulsed field gel electrophoresis and PCR amplification using primers which flank the breakpoint [13] followed by sequencing.

## MATERIALS AND METHODS

### Subjects

Clinical and hematological data for three β-thalassemia/HbE patients were collected, and blood was drawn from the patients and their parents and siblings for hematological examination and DNA analysis. The patients

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are part of a clinical service and were informed of the results of the diagnostic analysis.

### Hematological and Hemoglobin Analysis

Red cell indices were analysed by using a cell counter. Hemoglobin electrophoresis was carried out by using cellulose acetate gels (Helena Laboratories) in tris-ethylenediaminetetraacetic acid-borate buffer [14]. $HbA_2$ and HbE were determined by microcolumn (Biorad) and HbF by alkaline denaturation [15,16].

### DNA Isolation

Genomic DNA was isolated from 5 ml of blood collected in ethylenediaminetetraacetic acid by using the Progenome kit (Progen). Alternatively, 500 μl of blood was processed for PCR analysis after cell lysis and treatment with proteinase-K, 2 μl of this solution was added to the PCR reaction mix [17]. DNA for pulsed field gel electrophoresis was prepared from fresh lymphocytes immobilised and lysed in agarose blocks [18].

### β-Thalassemia Mutation Analysis

**ARMS.** Each DNA sample was first investigated by PCR amplification by using allele-specific priming (ARMS) [19] for seven known mutations previously described in the Indonesian population [20].

**Southern blotting.** Genomic DNA was digested with restriction enzymes, including *Ava*II, *Eco*RI, *Bam*HI, *Bgl*II, *Sac*I, *Xba*I, *Xmn*I. Globin DNA probes used for hybridization were 4.4 kb *Pst*I β, 2.3 kb *Pst*I δ and pRK29 [21]. After hybridization, membranes were washed at 65°C to a high stringency and autoradiographed with X-omat film at −70°C.

**PCR for Filipino deletion.** PCR amplification of the deletion breakpoint was performed by using the primers and PCR conditions described by Waye et al. [13].

**Sequencing of double-stranded PCR products.** The PCR products from the alleles with deletions were sequenced [22] by both forward and reverse primers using the cycling sequencing kit (Amersham).

**Pulsed field gel electrophoresis.** The block containing DNA from patients was digested with *Sfi*I restriction endonuclease [23]. The digests were separated in 1% FMC agarose by using the Chef-$DR^{+M}$ II electrophoresis cell (Biorad) for 24 hr at 200 V with pulse times ranging from 1 to 28 sec [24]. DNA was alkaline transferred to Hybond-$N^+$ and hybridized with the 2.3 kb *Pst*I δ-globin probe labeled by the random hexamer primer method [25].

### α-Thalassemia Deletion Analysis

**Southern blotting.** Genomic DNA was digested with *Bgl*II and *Bam*HI and hybridized with α- and ζ-globin probes [26].

### β-Globin Gene Cluster Polymorphisms and *Xmn*I Restriction Site

The *Xmn*I-$^{G}\gamma$ polymorphism was analysed by amplification of the $^{G}\gamma$-globin gene specific fragment, followed by digestion with 5–10 units of *Xmn*I endonuclease [27]. The β-globin gene cluster polymorphic sites *Hinc*II, 5′ to the ε-globin gene; *Hind*III, in IVSII of the $^{G}\gamma$- and $^{A}\gamma$-globin genes; *Hinc*II, within and 3′ to the ψβ-globin gene; and *Hinf*I and *Ava*II, respectively 5′ and within the β-globin gene, were analyzed similarly.

## RESULTS

### Clinical and Hematological Data

**Patients.** Patient A.II:2 was first seen at 7 years of age when he suffered from a viral infection. At that time his Hb level was 8 g/dl and his spleen was enlarged by 2 cm, but there were no Cooley's facies or growth retardation. After recovering from the infection his Hb level was maintained at around 10 g/dl without blood transfusion. At last examination, when he was 17 years old, he showed normal growth and development, no bone changes and a slightly palpable spleen.

Patient B.II:5 and patient C.II:1 have been dependent on blood transfusions since each was 5 years old. Both showed growth and development retardation, bone changes and excessive spleen enlargement. All of these symptoms have worsened because neither has been placed on a hypertransfusion regimen nor received iron chelation. The spleen size enlarged from 1 cm at diagnosis to 6 cm over 12 years for patient B.II:5 and from 6 cm to 12 cm over 6 years for patient C.II:1 The hematological results from all 3 patients at the time of diagnosis or before first transfusion are shown in Figure 1.

**Family studies.** The mother and one sibling of A.II:2 are β-thalassemia carriers with an unusually high $HbA_2$ (6.14%); two other siblings of the propositus were coincidently diagnosed with β-thalassemia/HbE. Neither had been diagnosed prior to this study, because there are no obvious clinical symptoms except a slightly low Hb level of 9 g/dl for A.II:1 and 11 g/dl for A.II:3.

Siblings of B.II:5 and C.II:1 who are β-thalassemia carriers also showed an unusually high $HbA_2$. All these individuals also synthesised markedly higher HbF than usual for β-thalassemia carriers. Figure 1 shows the pedigrees and hematological results from the families.

### β-Thalassemia Mutations

On initial DNA analysis, all patients appeared to be homozygous for Cd-26 (Glu →Lys), although their clinical and hematological parameters are not consistent with homozygosity for HbE. Each has an equal percentage of fetal hemoglobin and HbE, suggesting each is a compound heterozygote for $\beta^{o}$-thalassemia/HbE. The family

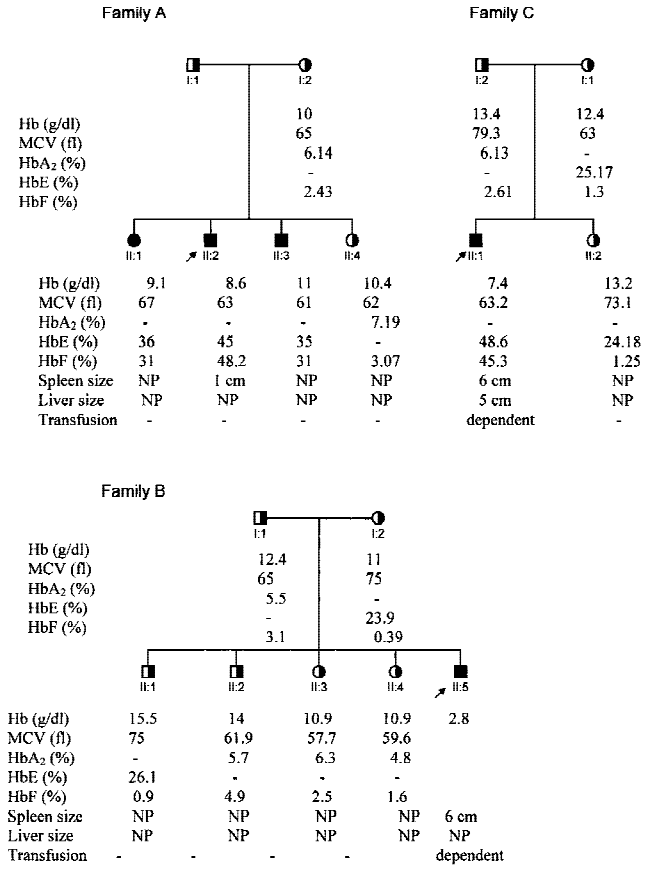


**Fig. 1. Pedigree and hematological results from families of patients with Filipino β°-thalassemia deletion/HbE. Arrows indicate the propositus. NP = non-palpable spleen or liver.**

studies showed unusually high $HbA_2$ in one parent in each family suggesting they are β-globin gene deletion carriers.

The DNA of the patients was examined using Southern blot analysis. DNA from the Filipino β°-thalassemia deletion patient examined by Motum et al. [10] was used as a control. All altered bands in the Southern blot were exactly the same as those in the positive control (Fig. 2).

Pulsed field gel electrophoresis shows a 140 kb fragment in the normal individual and 140 kb and 95 kb fragments in the patients. Based on this experiment the size of the deletion in Filipino β-thalassemia is 45 kb (Fig. 2).

PCR amplification using a primer pair flanking the deletion [13] generated a 376 bp fragment that is specific for the Filipino β°-thalassemia deletion. The non-deleted allele was amplified using the same forward primer and a different reverse primer located within the deletion producing a 482 bp fragment (Fig. 3). Sequencing analysis of the 376 bp fragment by using the forward primer showed the same sequences as those in the positive control (Motum) and the patient reported by Dimovski et al. [12] (results not shown).

### α-Globin Defects, *Xmn*I Restriction Site and β-Globin Gene Haplotypes

From Southern blot analysis, none of the patients showed either one or two α-globin gene deletion (results not shown).

The mild (A.II:2) and one of the severe (C.II:1) Filipino β°-thalassemia/HbE patients were heterozygous for the *Xmn*I restriction site, while the other severe patient (B.II:5) did not carry this $^{G}\gamma$-globin gene polymorphism. In both positive patients this polymorphism was carried by the Cd-26 mutant allele.

All three patients showed the same β-globin gene haplotype for the Filipino β°-thalassemia allele (+ − − − −). The last two polymorphisms (*Hinf*I and *Ava*II, 5′ and inside the β gene) in the seven polymorphism haplotype investigated are not detected as they are within the deletion. However, the patients showed different haplotypes for the Cd-26 mutant allele. Table I shows the β-globin gene cluster haplotypes and the *Xmn*I restriction site polymorphism results.

### Other Factors Influencing the Clinical Severity of β-Thalassemia

Post-transfusion malarial parasites (Plasmodium vivax) were transiently found in the peripheral blood film of both severe patients, but not in subsequent blood films examined 8 months later. All patients had a normal level of G6PD.

## DISCUSSION

Phenotypic variability has been detected in patients with β°-thalassemia/HbE. We find similar variability in patients who are compound heterozygous for HbE and a particular β°-thalassemia mutation common in Indonesia, the Filipino deletion.

The polymorphism (C-T) at −158 $^{G}\gamma$-globin gene which creates an *Xmn*I restriction site was reported as an ameliorating factor in β-thalassemia by increasing the synthesis of γ-globin chain [8], which binds the excess α-globin chains resulting in a high level of HbF and a resultant decrease in the degree of clinical severity.

However, in this study the patients who carry the heterozygous *Xmn*I restriction site have very different clinical severities, one being transfusion dependent and the other independent. This finding is similar to the results in a previous study [7] that showed a wide range of total Hb and HbF levels in patients carrying the heterozygous *Xmn*I restriction site (4 to 11 g/dl for total Hb and 2.02 to 7.27 g/dl for HbF). In comparison, the Hb level in all patients homozygous for the *Xmn*I restriction site was over 8.5 g/dl and the HbF level ranged from 3.92 to 7.35 g/dl. The variable influence of the heterozygous *Xmn*I

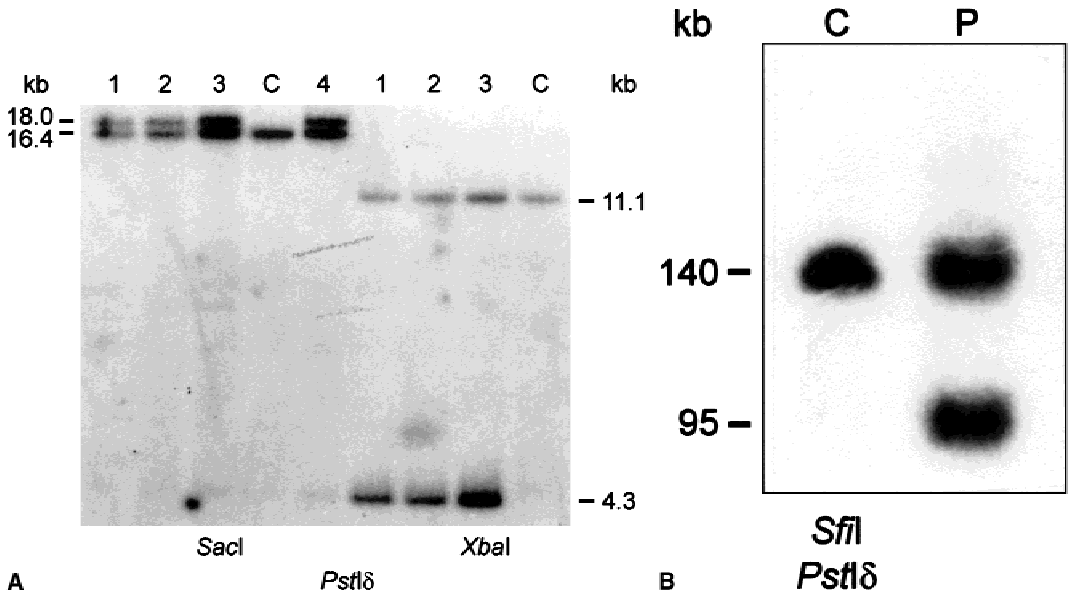


**Fig. 2. A: Southern blot of DNA from patients A.II:2, B.II:5 and C.II:1 (lanes 1–3), and a control positive patient with Filipino β°-thalassemia deletion (lane 4). C = control normal. All DNA samples were digested either with Sac I (left) or *Xba* I (right) and then hybridised to *Pst* I δ. B: Pulsed field gel electrophoresis of *Sfi* I digested DNA from a normal individual (C) and the patient A.II:2 with the heterozygous Filipino β°-thalassemia deletion (P).**

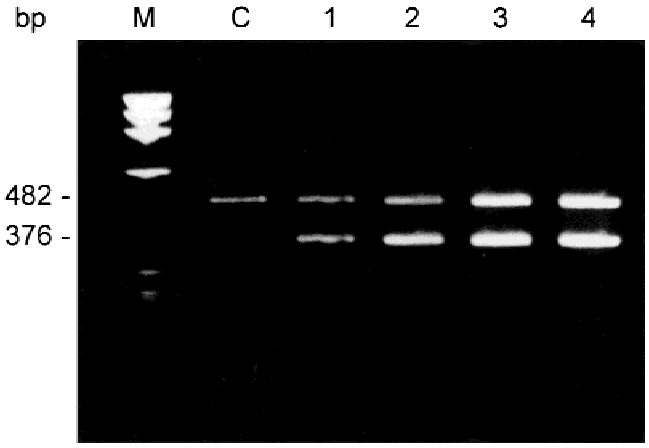


**Fig. 3. PCR amplification for the Filipino β°-thalassemia deletion using two sets of primers for normal and deleted alleles. Patients A.II:2, B.II:5, C.II.1 (lanes numbered 2–4) and the positive control (lane numbered 1) show both PCR fragments derived from the normal (482 bp) and deleted (376 bp) alleles. The normal individual (C) produces a 482 bp.**

**TABLE I. The *Xmn*I γ, β-Globin Gene Haplotypes and Clinical Severity in Patients With Filipino β-Thalassemia Deletion/HbE***

| Patients | Clinical severity | *Xmn*I-γ | $\beta^E$-globin allele haplotypes | Filipino $\beta^\circ$-globin allele haplotypes[a] |
|---|---|---|---|---|
| A.II:2 | mild | +/− | − + − − + + − | + − − − − |
| B.II:5 | severe | −/− | + − − − − + − | + − − − − |
| C.II:1 | severe | +/− | − + − + + + + | + − − − − |

*(+) Indicates the presence of restriction enzyme cleavage site and (−) its absence. In individuals heterozygous for the *Xmn*I polymorphism, the (+) allele cosegregates with the $\beta^E$-globin allele. The order of the sites 5′ to 3′ in β-globin gene cluster is as follows: *Hinc*II ε, *Hind*III$^G$γ, *Hind*III$^A$γ, *Hinc*II ψβ, *Hinc*II3′ ψβ, *Hinf*I3′β, *Ava*IIβ.

[a]The last two polymorphisms are within the deletion.

restriction site on decreasing the clinical severity of β-thalassemia was also reported by Thein et al. [6].

The − + − ++ β-globin gene cluster haplotype was reported as the common haplotype in β-thalassemia chromosomes from patients who did not need regular blood transfusion (clinically called β-thalassemia intermedia) [6,7]. All chromosomes carrying this haplotype also had the *Xmn*I restriction site. However, carrying this haplotype does not always mean the phenotype is mild; about 11% of β-thalassemia major patients also carry this haplotype [6]. Our patient, C.II:1, falls into the latter category as the haplotype is present but the patient is transfusion dependent.

Two ameliorating genetic factors, α-globin gene deletions and the β-globin gene haplotype (− + − ++), combined with the presence or absence of the *Xmn*I restriction site do not explain the phenotypic variation in our patients. It is unclear whether there are other genetic factors in family A which ameliorated the symptoms of patients A.II:2 and his siblings or other factors in family C which worsened the clinical course.

A specific sequence variation in the 5′ hypersensitive site-2 (HS-2) of the locus control region (LCR) of β-globin gene cluster has been associated with different levels of fetal hemoglobin [28]. In addition, sequence 5′ of the β-globin gene around position -540 were also associated

with variance in the level of HbF [29]. Neither factor was investigated in our study. The percentage of aberrant splicing in $\beta^E$ [9] was also not tested in our study.

The size of the Filipino deletion, 45 kb, is consistent with that reported by Motum et al. [10] rather than by Dimovski et al. [12], who reported a deletion larger than 105 kb based on dosage of probes on Southern blots in a heterozygous individual. We believe this patient carried the same deletion as that in our samples because the patient comes from the same country (Indonesia), and shows the same PCR products as well as the same sequencing results.

## CONCLUSIONS

The diversity in the clinical severity of β-thalassemia/HbE is important information for developing a thalassemia prevention program in Indonesia. Although not all known genetic factors that can contribute to the phenotypic diversity in β-thalassemia were examined here, the possibility remains that as yet undefined factors have to be invoked to explain the variation. This has been shown in other diseases [30,31] and indicates the importance of continuing to identify factors which explain this diversity, especially in the cases with the same genotype but expressing extreme ends of the clinical spectrum. This issue is critical for the geneticist when counseling for prenatal diagnosis.

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